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Polyvinyl chloride (PVC) plastic fragments release Pb additives that are bioavailable in zebrafish

David Boyle [a, b], Ana I. Catarino [a, c], Nathaniel J. Clark [b], Theodore B. Henry [a, d, *]

[a] The School of Energy, Geoscience, Infrastructure and Society, Heriot-Watt University, Edinburgh, EH14 4AS, UK
[b] School of Biological and Marine Sciences, University of Plymouth, Plymouth, PL4 8AA, UK
[c] Vlaams Instituut voor de Zee, Flanders Marine Institute, Wandelaarkaai 7, 8400, Ostend, Belgium
[d] Department of Forestry, Wildlife and Fisheries, and Center for Environmental Biotechnology, The University of Tennessee, Knoxville, TN, USA

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ABSTRACT

Plastic polymers such as polyvinyl chloride (PVC) may contain chemical additives, such as lead (Pb), that are leachable in aqueous solution. The fragmentation into microplastics (MPs) of plastics such as PVC may facilitate desorption of chemical additives and increase exposure of aquatic animals. In this study, the role of chemical additives in the aqueous toxicity of PVC, high-density polyethylene (HDPE) and polyethylene terephthalate (PET) MPs were investigated in early-life stage zebrafish (*Danio rerio*) by assessment of changes in expression of biomarkers. Exposure of zebrafish larvae to PVC for 24 h increased expression of *metallothionein 2* (*mt2*), a metal-binding protein, but no changes in expression of biomarkers of estrogenic (*vtg1*) or organic (*cyp1a*) contaminants were observed. HDPE and PET caused no changes in expression of any biomarkers. A filtered leachate of the PVC also caused a significant increase in expression of *mt2* and indicated that a desorbed metal additive likely elicited the response in zebrafish. Metal release was confirmed by acid-washing the MPs which mitigated the response in *mt2*. Metal analysis showed Pb leached from PVC into water during exposures; at 500 mg PVC L^{-1} in water, 84.3 ± 8.7 μg Pb L^{-1} was measured after 24 h. Exposure to a Pb-salt at this concentration caused a comparable *mt2* increase in zebrafish as observed in exposures to PVC. These data indicated that PVC MPs elicited a response in zebrafish but the effect was indirect and mediated through desorption of Pb from PVC into the exposure water. Data also indicated that PVC MPs may act as longer-term environmental reservoirs of Pb for exposure of aquatic animals; the Pb leached from PVC in 24 h in freshwater equated to 2.52% of total Pb in MPs leachable by the acid-wash. Studies of MPs should consider the potential role of chemical additives in toxicity observed.

1. Introduction

The release of plastic debris into the environment has led to their near ubiquity in the aquatic environment and constant routes of exposure for many organisms including humans. Awareness of this issue has garnered public attention and heightened demands for sustainable alternatives to plastic technologies and solutions to remedy widespread plastic pollution for the benefit of aquatic organisms and humans (Catarino et al., 2018). Of particular concern is the presence of micro- and nano-plastics < 5 mm in size. These have been documented throughout the aquatic environment including remote locations (e.g. the Arctic (Peeken et al., 2018)), and in aquatic fauna (e.g. in fish larvae (Steer et al., 2017)), in seafood (e.g. mussels (Catarino et al., 2018)), and in faeces of humans (Schwabl et al., 2019). With projections of one trillion plastic pieces in the aquatic environment by 2050 and estimates of human consumption of plastic exceeding 10,000 particles $year^{-1}$ (Catarino et al., 2018), there is an urgent need to characterise the effects of MP exposure at all trophic levels to identify priorities for further actions.

Fish ingest MPs (Steer et al., 2017) and may do so both incidentally (Critchell and Hoogenboom, 2018) and deliberately, mistakenly identifying them as food (Khan et al., 2015). Humans are also exposed to MPs both through the consumption of contaminated seafood and from airborne fibres (Catarino et al., 2018; Dris et al., 2017; Schwabl et al., 2019). There is no evidence of effects

* Corresponding author. The School of Energy, Geoscience, Infrastructure and Society, Heriot-Watt University, Edinburgh, EH14 4AS, UK.
E-mail address: t.henry@hw.ac.uk (T.B. Henry).

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in humans, but gut lesions have been attributed to MP exposure in European seabass (*Dicentrarchus labrax*; Pedà et al., 2016). Impacts on internal tissues have also been reported in some studies in fish but there is considerable divergence in the magnitudes of effects observed and a consensus position on the toxicity of MPs is yet to emerge from the literature. For example, MPs were reportedly neurotoxic in *D. labrax* and caused an inhibition of acetyl cholinesterase activity in the brain (Barboza et al., 2018); but, in other studies, no effects in fish have been observed (e.g. Jovanović et al., 2018). Whether this difference in reported effects is attributable to the dose, size, shape or chemistry of the MPs or limitations in study design is unclear and warrants further investigation. This is also important to the understanding of effects of MPs in humans due to the role of diet as a route of MP exposure (Catarino et al., 2018; Schwabl et al., 2019) and the use of fish assays to model human exposure to toxicants.

Microplastics are often associated with other environmental contaminants. These may include metals, pharmaceuticals and polycyclic aromatic hydrocarbons that become sorbed to MP surfaces in aqueous environments (see review by Teuten et al., 2009). For example, silver (Ag) sorbed to polyethylene microbeads at concentrations exceeding 10 μmol g^{-1} after 18 h in artificial freshwater (Khan et al., 2017). Additionally, chemical additives that may present a risk to human and environmental health are often incorporated into plastics during synthesis (Teuten et al., 2009). For example, synthesis of polyvinyl chloride (PVC) can require thermal stabilisers including metals, lead (Pb), barium (Ba) and tin (Sn) compounds, or organic compounds such as phthalates (Hahladakis et al., 2018). While the release of co-contaminants and chemical additives from surfaces of plastics is an existing health concern, especially into potable water, fragmentation of plastics into microscale particles with high surface to volume ratios has the potential to increase the rate of release and alter exposure pathways in humans and aquatic animals.

Although the bioavailability of microplastics is considered low at epithelia because they are excluded from transmembrane transport by virtue of their large size (Lu et al., 2016), ingestion of environmental microplastics may increase delivery and release of greater quantities of sorbed co-contaminants at sensitive epithelia e.g. the gastrointestinal tract of animals (Khan et al., 2015). For example, Lu et al. (2018) demonstrated increased accumulation of cadmium (Cd) at the gill and gut of zebrafish exposed to polystyrene beads. This could then contribute to toxicological responses observed in field-exposed fish (e.g. Alomar et al., 2017). It has been shown that the rate of desorption of metals from microplastic surfaces can be increased in higher ionic strength biological fluids such as gut saline, or at acid pH characteristic of the fish stomach, compared to freshwaters (Khan et al., 2017).

In the present study, the aim was to investigate the effects of PVC MPs. Despite proposed restrictions on the use of Pb in PVC in the European Union (EU) due to the human and environmental hazards Pb presents (see reviews by Boskabady et al., 2018; Mager, 2012), the import of PVC containing Pb compounds to the EU from other jurisdictions with no such restrictions is expected to continue thereafter (European Chemicals Agency, 2016). PVC is also abundant in field-collected samples of plastic debris (Munier and Bendell, 2018) and has been documented in human faeces (Schwabl et al., 2019), and these data indicate that there is probable exposure of humans to Pb additives in PVC. Here, we used trace metal analyses to measure the release of metals from PVC. We also measured changes in gene expression, especially *metallothionein 2* (*mt2*), a metal binding protein, but also biomarkers of organic and estrogenic xenobiotics, to investigate the bioavailability of leached additives in early-life stage zebrafish, an exposure model.

2. Materials and methods

2.1. Overview of study

A series of experiments were devised to investigate the toxicity of PVC microplastics in larval zebrafish (section 2.4). Gene expression was used as an indicator of bioavailability (section 2.5) and trace metals were measured using ICP-MS (section 2.6). Finally, the effects of PVC in zebrafish were compared to exposures to other microplastics, high-density polyethylene (HDPE) and polyethylene terephthalate (PET).

2.2. Zebrafish husbandry

Brood stock zebrafish (WIK) were maintained in a recirculating water system located in a designated temperature controlled (27 ± 1 °C) aquarium facility at Heriot-Watt University, Edinburgh, UK. The artificial lighting had a photoperiod of 14:10 h light: dark. The water used for culturing stock fish and which was also used in all experiments (the aquarium water) was formulated from reagent grade salts added to reverse osmosis purified water. The final concentrations of salts in the water were: 0.294 mg L^{-1} $CaCl_2 \cdot 2H_2O$, 0.123 mg L^{-1} $MgSO_4 \cdot 7H_2O$, 0.0647 mg L^{-1} $NaHCO_3$, 0.0057 mg L^{-1} KCl, and the water was at pH 8.0. Water quality was routinely monitored (e.g. for total ammonia, nitrate and nitrite) and part-refreshed on a twice weekly basis or more frequently if required. Fish were fed newly hatched brine shrimp *Artemia* spp. and a commercial zebrafish diet (ZM Fish Food, ZM Systems, Winchester, UK), daily.

To obtain larvae for experiments, pairs of zebrafish (one male and one female) were netted from stock tanks and gently transferred to 1 L breeding tanks (Mbk Installations Ltd, Nottingham, UK) fitted with a partition to separate the fish. Fish were then left overnight. The following morning, the water in the breeding tanks was refreshed, the partitions were removed, and fish proceeded to spawn. Approximately 60 min later, the adult fish were transferred back to stock tanks and all embryos from multiple spawning pairs were pooled and distributed between Petri dishes at a density of approximately 50 embryos $dish^{-1}$. Zebrafish were left to develop until 72 h post-fertilisation (hpf) and hatched larvae were then used in experiments. At this stage of development zebrafish are not free feeding i.e. before being licenced in the UK and only the number of larvae necessary for robust statistical analyses were used.

2.3. Microplastics and $Pb(NO_3)_2$ stock solution

The PVC, HDPE and PET microplastics used in this study were kind gifts from Dr Chelsea Rochman, The University of Toronto, Canada. The PVC and PET microplastics originated from Plastic Industry Development Centre, Taiwan, R.O.C., and the HDPE originated from The Dow Chemical Company, Michigan, USA. The microplastic fragments were subsequently sieved to obtain a plastic fraction of approximately 200 μm of each material that would be used in experiments. An image of the PVC microplastics and a particle size distribution calculated using ImageJ software (Schindelin et al., 2012) are shown in Supplemental Fig. 1. The sizes of $n = 25$ PVC particles were 152.4 ± 37.6 μm (mean ± standard deviation) and the sizes of $n = 30$ HDPE particles and $n = 30$ PET particles were 297.9 ± 51.6 μm and 257.7 ± 67.4 μm, respectively. A stock solution of $Pb(NO_3)_2$ (Sigma-Aldrich, UK) was prepared in ultrapure water (Milli-Q, Merck Millipore, UK) in a glass scintillation vial at a concentration of 1 g Pb L^{-1}.

2.4. Experimental series of zebrafish exposures

In each experiment, zebrafish larvae at 72 hpf were collected from Petri dishes with a transfer pipette and placed into acid-washed (5% HNO_3) 50 mL glass beakers. Each beaker (the experimental replicate, $n = 3$ treatment^{-1}) contained 20 larvae. The water transferred with larvae to the beakers was then carefully removed and replaced with 40 mL of aquarium water spiked with microplastics or dissolved Pb (see subsections 2.4.1–2.4.5, water chemistry and conditions of exposure were as described for adult zebrafish in section 2.2). Controls were included in each experiment and were treated as detailed above for the treatment groups but with the exception that the aquarium water contained neither microplastics nor Pb. After 24 h of exposure, larvae from each beaker were pooled into a microcentrifuge tube, the exposure water including any microplastics were removed with a pipette tip and the tubes containing larvae were then immediately stored at −80 °C for later gene expression analyses (section 2.5). No treatment dependent mortality of zebrafish was observed during the exposures.

2.4.1. Experiment 1

In experiment one, the dose-dependent effects of PVC microplastics exposure were investigated by exposing zebrafish larvae to 0, 125, 250 and 500 mg L^{-1} of PVC. The concentrations chosen were similar to those used in previous MP co-contaminant sorption studies (i.e. 400 mg L^{-1} used by Bakir et al., 2012; Sleight et al., 2017; Teuten et al., 2007). The concentration of 500 mg PVC L^{-1} approximates to 195,496 MP L^{-1} (if MPs were assumed to be spherical). The MPs were added directly to water in the beakers to start the exposures; nothing was added to control beakers. After 24 h, when larvae were collected, water samples (2 mL) were also collected for trace metal analyses (section 2.6).

2.4.2. Experiment 2

The aim of experiment two was to investigate the contribution of thermal stabilisers released from PVC to gene expression profiles in fish. The PVC (500 mg L^{-1}) was pre-incubated in aquarium water for 24 h at 27 ± 1 °C before being removed by filtering the water (leachate) through a 10 μm filter (CellTrics, Sysmex, UK). This PVC was then discarded. Fish were then exposed to either control, PVC (dry material added directly to beaker) or the leachate for 24 h.

2.4.3. Experiment 3

To further investigate the role of substances released from the surface of the PVC to the responses measured in zebrafish, the PVC (20 mg) was pre-washed with either ultrapure water, 2% HNO_3 (Trace Analysis grade, Fisher, UK), 2% Neutracon (a detergent, Decon Laboratories, UK), or 100% ethanol (Molecular Biology grade, Fisher, UK), prior to the exposures. Specifically, PVC was vortexed in the wash solution and then incubated for 1 h at room temperature. The PVC was then collected in a 100 μm CellTrics filter and then the wash solution removed with an excess of ultrapure water before the PVC was added directly to beakers containing zebrafish for a final concentration of 500 mg L^{-1}.

2.4.4. Experiment 4

In experiment four, zebrafish were exposed to HDPE and PET at concentrations of 500 mg L^{-1} and the expression of biomarker genes was measured.

2.4.5. Experiment 5

The aim of experiment five was to investigate the significance of Pb released from PVC to observed effects on gene expression in zebrafish. To do this, larvae were exposed to a Pb salt $Pb(NO_3)_2$ at nominal Pb concentrations of 0, 0.033, 0.33, 3.3 and 33 mg L^{-1}. These concentrations were chosen to match the maximum possible Pb concentrations leaching from PVC microplastics (see sections 3.2 and 3.3). Water samples (2 mL) were taken from just below the water surface immediately after dosing to measure Pb concentrations (section 2.6).

2.5. Gene expression analyses

Expression of genes that are biomarkers of the bioavailability of metals, *mt2*, environmental estrogens, *vitellogenin 1* (*vtg1*), and organic xenobiotics, *cytochrome p450 1a* (*cyp1a*), were measured in zebrafish larvae sampled from experiments 1–5 (see section 2.4). In this study, we defined bioavailability as the extent to which a compound reacts with biological molecules in a tissue (Semple et al., 2004). In zebrafish larvae, a model organism, changes in expression of biomarkers to assess the bioavailability of xenobiotics are well characterised (e.g. *mt2*, Henry et al., 2013; *vtg1*, Park et al., 2010). Total RNA from pooled samples of zebrafish larvae that were homogenised with a motor-driven hand homogeniser was isolated using an RNeasy Mini Plus Kit and following the manufacturer's instructions (Qiagen, UK). On column treatment of samples with DNase (RNase-Free DNase Set, Qiagen, UK) eliminated any genomic DNA contamination. Total RNA was eluted in ultrapure nuclease-free water (Ambion, Thermo Scientific, UK) and the concentration and presence of impurities assessed through spectrophotometry (NanoDrop, ND-1000, Thermo Fisher Scientific, UK). The synthesis of cDNA was carried out using the Precision nanoScript 2 Reverse Transcription Kit (Primer Design, UK) in 10 μL reactions containing 2 μg RNA and oligo dT primers according to the manufacturer's instructions.

Quantitative PCR was performed with cDNA diluted 1 in 25 with nuclease-free water and using a light cycling PCR machine (Applied Biosystems StepOne System, Thermo Fisher Scientific, UK) with 20 μL samples containing 300 nM gene specific primers (Table 1) and a SYBR green qPCR mastermix (Precision PLUS qPCR Master Mix, Primer Design, UK). The conditions were as follows: 2 min of initial enzyme activation at 95 °C followed by 40 cycles of denaturation at 95 °C for 10 s and data collection at 60 °C for 60 s. All samples were analysed in triplicate with appropriate no-template controls included in each run and with dissociation analysis performed within the qPCR run to verify the specificity of the primer pair. The relative copy number of mRNA transcripts was calculated according to a Ct-based relative quantification after normalisation to expression of *ribosomal protein L8* (*rpl8*). Data are shown normalised to expression in control treatment groups using the delta-delta Ct fold change calculation.

2.6. Trace metal analyses

Total leachable concentrations of trace metals in PVC MPs, and Pb in water samples from exposures of zebrafish to PVC and Pb as $Pb(NO_3)_2$ were measured using an iCAP RQ ICP-MS (Thermo Scientific, UK). To measure the total leachable metal fraction from PVC microplastics, 20 mg PVC (equal mass as used in exposures, $n = 3$ replicates) was incubated in 5 mL of 2% HNO_3 for 1 h, after which the PVC MPs were removed in a CellTrics filter and metals measured in the filtrate. Water samples collected from beakers containing zebrafish larvae exposed to different concentrations of PVC (experiment one, $n = 3$) and to $Pb(NO_3)_2$ (experiment five, $n = 1$) were acidified with 0.5 mL HNO_3 (Trace Analysis grade, Fisher, UK) prior to analysis. Concentrations of metals in samples were compared to analytical standards (Fisher, UK) and these standards were also analysed frequently throughout the analytical run to monitor the instrument for drift. A reference water sample

Table 1
Gene specific primers used in early life-stage zebrafish.

Gene	Accession #	Primer Sequence (5' – 3')	Amplicon (bp)	Source
metallothionein 2 (*mt2*)	NM_001131053.2	Forward: TGTTCTCAATCTTGTCTGTTTAATG Reverse: ACATCTCGTGATAGTCTTATTTGC	108	1
vitellogenin 1 (*vtg1*)	NM_001044897.3	Forward: ATCAGTGATGCACCTGCCCAGATTG Reverse: ACGCAAGAGCTGGACAAGCTGAA	117	2
cytochrome P450 1a (*cyp1a*)	NM_131879.2	Forward: AGGACAACATCAGAGACATCACCG Reverse: GATAGACAACCGCCCAGGACAGAG	174	2
ribosomal protein L8 (*rpl8*)	NM_200713.1	Forward: CCGAGACCAAGAAATCCAGAG Reverse: CCAGCAACAACACCAACAAC	91	3

Source: 1) PrimerDesign, Camberley, UK; 2) Aguirre-Martínez et al. (2017); 3) Laing et al. (2016).

(*Enviro*MAT Drinking Water, Low (EP-L); Qmx Laboratories, UK) was also included in the analytical run to verify the accuracy of the measurements; the measurement for Pb was within the tolerance interval of the reference sample.

2.7. Data handling and statistical analyses

All data presented are means ± standard deviation (SD). All statistical tests were performed in SigmaPlot (*v.* 13.0, Systat Software Inc., San Jose, USA). All data were tested for normality (Shapiro-Wilk test) and equality of variances (Brown-Forsythe test) and if not normally distributed were $\log_{10}$ transformed. Statistically significant differences between datasets were detected using one-way ANOVA with Holm-Sidak test *a posteriori*, or Student's *t*-test. A p value of <0.05 was considered significant. Data of the effects of Pb as $Pb(NO_3)_2$ on *mt2* expression were curve-fitted with a 2-parameter hyperbola.

3. Results

3.1. Gene expression responses in zebrafish to microplastic exposures

Exposure to PVC microplastics resulted in a dose-dependent increase in expression of *mt2* (Fig. 1A; one-way ANOVA, $p = 0.007$). This culminated in a significant 2.1-fold increase in *mt2* expression after exposure to 500 mg PVC L^{-1} when compared to controls (Holm-Sidak test, $p = 0.01$). In contrast, exposure to the highest concentration of PVC microplastics (500 mg L^{-1}) had no significant effect on expression of either *vtg1* or *cyp1a* compared to controls (Student's *t*-tests, $p = 0.13$ and $p = 0.29$, respectively; Fig. 1B).

The response of zebrafish to additives leached from the PVC MPs was explored by exposing larvae to filtered aquarium water that had been pre-incubated with 500 mg L^{-1} PVC MPs for 24 h (Fig. 2A). This leachate caused an upregulation in *mt2* expression (one-way ANOVA, $p < 0.001$) that was greater than in both controls and in fish exposed to untreated PVC microplastics (Holm-Sidak tests, $p < 0.001$ and $p = 0.02$, respectively). The role of leached chemical additives in the response of zebrafish to PVC was further explored by washing the PVC MPs prior to exposures (Fig. 2B). This approach significantly altered the responses in zebrafish larvae, but this was dependent on the wash solution used (one-way ANOVA, $p < 0.001$). Compared to controls, an upregulation of *mt2* was measured in larvae exposed to PVC pre-washed in ultrapure water (Holm-Sidak test, $p < 0.001$). A comparable increase in expression of *mt2* was also measured in larvae exposed to PVC washed in either ethanol or neutracon (Holm-Sidak tests, both $p < 0.001$). In contrast, pre-washing the microplastics with 2% HNO_3 ameliorated this effect of PVC on gene expression. Expression of *mt2* was not significantly different in zebrafish exposed to acid-washed PVC as compared to controls ($p = 0.522$) and expression was significantly lower than in response to PVC washed with water, ethanol or neutracon (Holm-Sidak tests, all $p < 0.001$).

Expression of biomarkers was also investigated in zebrafish larvae exposed to HDPE and PET. Compared to controls, neither

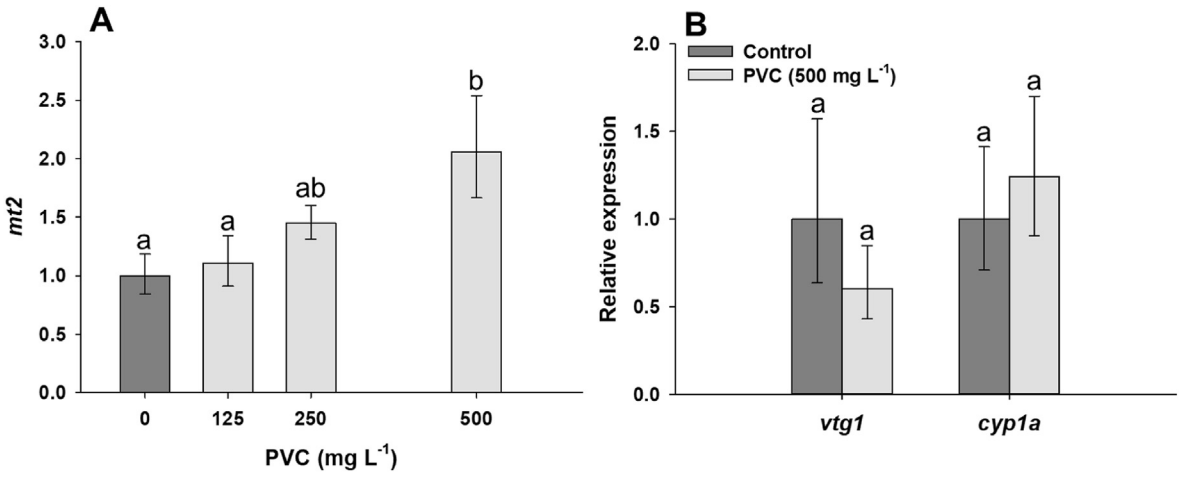

Fig. 1. Relative expression of A) *metallothionein 2* (*mt2*) and, B) *vitellogenin 1* (*vtg1*) and *cytochrome P450 1a* (*cyp1a*), in zebrafish larvae exposed for 24 h to polyvinyl chloride (PVC) microplastics in aquarium water. Expression of *vtg1* and *cyp1a* were measured in larvae exposed to PVC at 500 mg L^{-1}, only. Expression was normalised to control larvae (dark bars) that were not exposed to PVC. Data are means ± SD ($n = 3$). Different lower case letters indicate significant differences between treatment groups (one-way ANOVA with post-hoc Holm-Sidak test, Student's *t*-tests, $p < 0.05$).

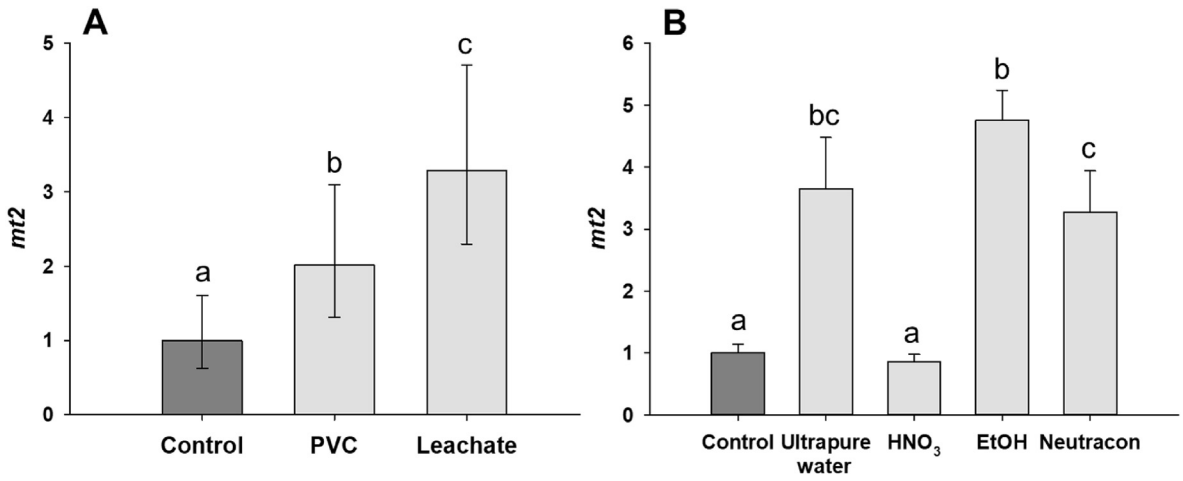

Fig. 2. Relative expression of *metallothionein 2* (*mt2*) in zebrafish larvae exposed for 24 h to A) 500 mg L^{-1} PVC microplastics, or, a filtered leachate from 500 mg L^{-1} PVC microplastics incubated in aquarium water for 24 h and, B) 500 mg L^{-1} PVC washed for 1 h prior in ultrapure water, 2% nitric acid (HNO_3), 100% molecular-grade ethanol (EtOH) or 2% detergent (Neutracon) before addition to aquarium water for exposures with larvae. Expression was normalised to control larvae (dark bars) in aquarium water containing neither PVC nor leachate. Data are means ± SD ($n = 3$). Different lower case letters indicate significant differences between treatment groups (one-way ANOVAs with post-hoc Holm-Sidak test, $p < 0.05$).

500 mg L^{-1} HDPE nor 500 mg L^{-1} PET caused significant changes in expression of *mt2*, *cyp1a* or *vtg1* expression in zebrafish (Fig. 3; one-way ANOVAs, $p = 0.340$, $p = 0.497$ and $p = 0.522$, respectively).

3.2. Trace metals in PVC

Incubation of PVC MPs in 2% HNO_3 liberated trace metals, especially Pb. Concentrations of Pb were calculated to be 6691 ± 388 ng mg^{-1} PVC which equated to 0.67 ± 0.04% of the PVC on a per mass basis. Acid also liberated low, but measurable, quantities of Ba, Cd, Cu, Fe, Mn and Zn (0.2–39.0 ng mg^{-1} PVC). In exposures with zebrafish in water, some of this Pb was released from the PVC. Measured concentrations of Pb in water were 20.2 ± 2.7, 46.0 ± 4.7 and 84.3 ± 8.7 μg L^{-1} after 24 h exposures to 125, 250 and 500 mg L^{-1} PVC microplastics, respectively. At the highest concentration of PVC used, 500 mg L^{-1}, this concentration of Pb released in water equates to 2.52% of Pb liberated by the acid-wash. The measured background concentration of Pb in controls was 0.05 μg L^{-1}.

3.3. Exposure to $Pb(NO_3)_2$

Measured concentrations of Pb were consistently lower than the nominal values. Precipitation of Pb was visible in beakers at the highest concentrations of Pb tested i.e. 3.3 and 33 mg L^{-1} and indicated the exposure was at the solubility limits for Pb. Nevertheless, exposure to $Pb(NO_3)_2$ resulted in a dose-dependent increase in *mt2* expression up to a nominal concentration of 3.3 mg L^{-1} (measured concentration of 1.58 mg L^{-1}). These expression data are shown in Fig. 4 plotted against measured Pb concentrations in water and curve-fitted with a two-parameter hyperbola ($r^2 = 0.96$, $p < 0.001$). Expression of *mt2* (E_{mt2}) in

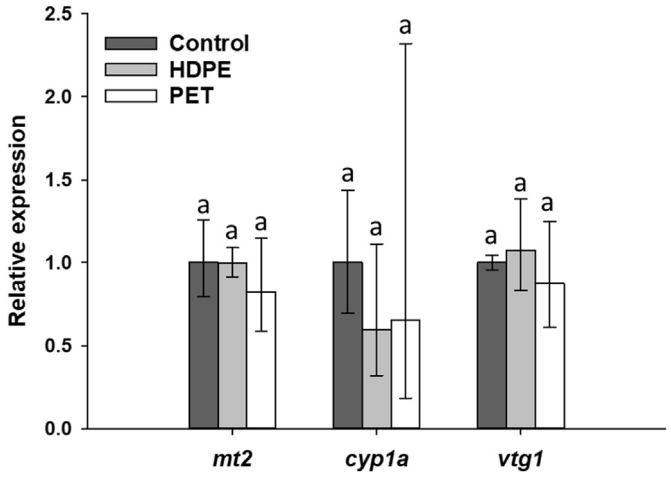

Fig. 3. Relative expression of *metallothionein 2* (*mt2*), *cytochrome P450 1a* (*cyp1a*) and *vitellogenin 1* (*vtg1*) in zebrafish larvae exposed for 24 h to 500 mg L^{-1} of high-density polyethylene (HDPE) or polyethylene terephthalate (PET) microplastics in aquarium water. Expression was normalised to control larvae (dark bars) that were exposed to neither HDPE nor PET. Data are means ± SD ($n = 3$). Different lower case letters indicate significant differences between treatment groups (one-way ANOVA with post-hoc Holm-Sidak test, $p < 0.05$).

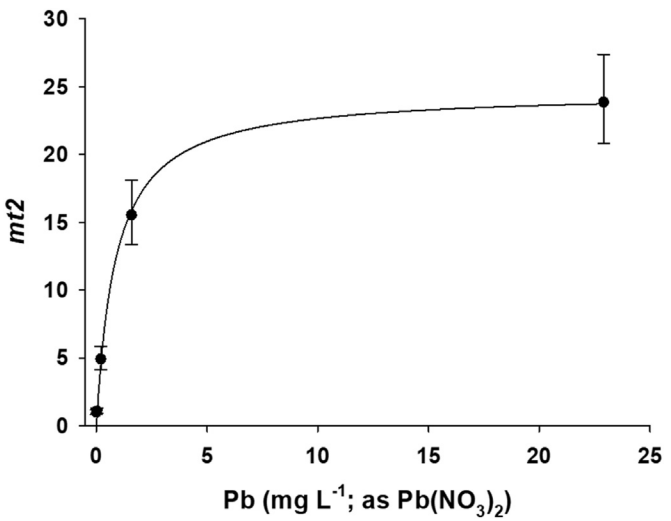

Fig. 4. Relative expression of *metallothionein 2* (*mt2*) in zebrafish larvae exposed for 24 h to lead (Pb, as $Pb(NO_3)_2$) in aquarium water. Data are means ± SD ($n = 3$). Data were curve-fitted with a two-parameter hyperbola ($r^2 = 0.96$, $p < 0.001$) and expression of *mt2* (E_{mt2}) in zebrafish exposed to concentrations of Pb (C_{Pb}; mg L^{-1}) was derived using $E_{mt2} = (24.9 \times C_{Pb})/(0.89 + C_{Pb})$.

zebrafish exposed to concentrations of Pb (C_{Pb}; mg L^{-1}) was derived using $E_{mt2} = (24.9 \times C_{Pb})/(0.89 + C_{Pb})$. At a soluble Pb concentration of 84.3 μg L^{-1} i.e. the mean concentration of Pb measured in water after 24 h exposure to 500 mg PVC L^{-1} (see section 3.2), a 2.16-fold increase in expression of *mt2* compared to controls would be predicted if expression of *mt2* is attributable to Pb released during exposure to 500 mg PVC L^{-1}. This value was close to the 2.06 fold increase in expression of *mt2* in response to exposure to 500 mg L^{-1} PVC.

4. Discussion

In this study, exposure to PVC MPs caused an increase in expression of *mt2* in zebrafish larvae. This response was mitigated by washing the PVC in acid prior to exposures with zebrafish. Trace metal analysis of this acid wash solution revealed the presence of a labile Pb additive in PVC that was also released into water during exposures in zebrafish. Exposure of zebrafish to soluble Pb as $Pb(NO_3)_2$ at the concentration released from PVC in water was then demonstrated to induce *mt2* expression at a level comparable to that measured in exposures with PVC MPs. Altogether, these data indicated that PVC MPs could elicit a response in zebrafish but that the effects were indirect and attributable to a bioavailable Pb fraction released from the MPs and not the particles themselves.

Exposure of early life-stage zebrafish to PVC MPs for 24 h caused a dose-dependent increase in expression of *mt2*. At this stage of development the zebrafish larvae had a yolk sac and were not free-feeding (mouth not fully developed), and MPs were unlikely to be ingested. Rather, and since the PVC MPs used in this study are too large for transport across epithelia in fishes (≥20 μm; Lu et al., 2016), a response attributable to the particle would be expected to be mediated by the interaction of PVC at the surface of epithelia. Previously, exposure to polymer microspheres has been shown to increase lipid peroxidation in gills of *D. labrax* (Barboza et al., 2018) and *mt2* has been shown to be responsive to oxidative stress (Chiaverini and De Ley, 2010). However, oxidative injury mediated by an epithelia-particle interaction appears not to be relevant to the increase in *mt2* observed in the present study; this effect is better explained by a labile co-contaminant or PVC additive, and most likely a metal, released from the PVC during exposures with zebrafish. This was because a filtered leachate from PVC MPs caused an increase in *mt2* expression in zebrafish. Moreover, the response to the leachate was greater than measured in zebrafish exposed to PVC. This result is explained by the expected dynamics of aqueous contaminant release from PVC. After dosing the test water with PVC, the co-contaminant would be expected to be gradually released over the 24 h test period. In contrast, the leachate contains the maximum concentration of the contaminant after 24 h incubation of PVC in water.

Metal analysis of samples of the acid-wash of PVC and of water samples taken from vessels used in zebrafish exposures, revealed the presence of a Pb additive in the plastic and its dose dependent dissociation from PVC into water at pH 8.0. Lead is an environmental contaminant of concern. In humans, Pb exposure has been linked to impaired cognitive function and cancers (Anttila et al., 1995; Jusko et al., 2008). In fish, Pb has been shown to cause hypocalcemia and disturbances in neurotransmitter systems, among other effects reported (see review by Mager, 2012). Unlike the PVC MPs used in the present study, dissolved Pb is bioavailable in fish and uptake from water occurs via shared Ca^{2+} pathways across membranes of ionocytes (Rogers and Wood, 2004). Once in the cell, Pb has been shown to induce expression of metallothionein genes e.g. in rockfish (*Sebastes schlegelii*; Kim and Kang, 2017). This response was also evident in the present study that showed an increase in *mt2* in zebrafish exposed to $Pb(NO_3)_2$. Furthermore, there was strong agreement between the concentration of Pb measured in water during exposures to PVC and predicted *mt2* response in zebrafish from this dose-response curve.

The role of Pb in the observed effect of PVC was further confirmed by exposing zebrafish to acid washed MPs. In contrast to the pre-treatment of MPs with water, ethanol or a dilute surfactant, acid washing the PVC mitigated the subsequent *mt2* response in zebrafish (competitive interactions with high $[H^+]$ at binding sites on PVC liberated Pb^{2+} into the wash solution). This effect of a 1 h acid wash on Pb leaching from PVC also has relevance to the predicted behaviour of PVC in biological systems. After 24 h, an estimated 2.5% of the total acid-labile fraction of Pb was released into water from the surface of PVC. This indicates that the PVC may serve as a source of Pb for longer than the 24 h used in the present study. Humans and fish have been shown to ingest microplastics (e.g. Catarino et al., 2018; Khan et al., 2015; Schwabl et al., 2019). The acid environment of the stomach of fish has also been shown to liberate MP-sorbed metal co-contaminants. For example, Khan et al. (2017) demonstrated sorption of aqueous Ag to polyethylene MP beads at circumneutral pH in freshwater, but rapid (within 1 h) release of sorbed Ag from PE in synthetic gut saline at pH < 4.1. This suggests that ingestion of PVC could lead to the liberation of Pb in the vertebrate stomach and exposure via the gastrointestinal tract.

The response of zebrafish to acid-washed PVC also suggested that the MPs were of negligible toxicity once the Pb additive was removed from exposures. Although a comprehensive toxicity assessment of PVC MPs in zebrafish was not performed in this study, biomarkers of estrogens, organic chemicals, metals and oxidative stress were unaffected by acid-washed PVC microplastics in zebrafish. Furthermore, HDPE and PET of comparable size to the PVC elicited no change in expression of *mt2*, *vtg1* or *cyp1a* in zebrafish. These results could indicate that plastic particulates may not pose a hazard to zebrafish larvae from external exposure at epithelia. These data are in agreement with some previous studies. For example, Karami et al. (2017) reported no perturbations in biomarkers of toxicity in zebrafish larvae exposed to pristine low-density polyethylene fragments (≤0.5 mg L^{-1}, 90% < 17.6 μm). The low-density polyethylene fragments used also had no measurable associated metals, phthalates or organic pollutants (Karami et al., 2017). In contrast, Qiang and Cheng (2019) reported decreased swimming ability and oxidative stress in larval zebrafish that was associated with the accumulation of green fluorescent polystyrene microspheres (1 mg L^{-1}, 1 mm). These differences in toxicity measured in early-life stage zebrafish could be due to the different chemistry, morphology and/or behaviours of MPs in suspension that could alter the dynamics of MP exposure. For example, it has been suggested that freshly milled plastic pellets with irregular surfaces may be more toxic than weathered and smoothed pellets used in other studies (Jovanović et al., 2018).

The PVC MPs used in the present study are representative of fragments of industrial products that are discarded into the aquatic environment (Munier and Bendell, 2018). The use of chemical additives in plastics is commonplace and includes not only metals, but also other known toxicants such as flame retardants and phenolic compounds; estimates of between 35 and 917 tonnes of organic plastic additives may enter the oceans through leaching, annually (Suhrhoff and Scholz-Böttcher, 2016). Without adequate experimental controls for chemical additives this could complicate the interpretation and comparability of data gathered from laboratory toxicity tests. This paradigm also extends to field observations attempting to correlate field exposure to MPs and biomarkers of toxicity in wild caught fish (e.g. Alomar et al., 2017); as discussed above, approximately 2.5% Pb leached from the PVC in 24 h and the

PVC may serve as a reservoir of Pb for an extended duration of time in freshwater. A minimum standard of MP characterisation should therefore be expected by peer-reviewers before the publication of data is recommended. Previously, metal catalysts used in the synthesis of engineered carbon nanomaterials were shown to be the source of toxicity in testing of these carbon materials with aquatic animals, effects that had been previously attributed to the carbon material itself (Pulskamp et al., 2007). This is a barrier to the advancement of the science.

5. Conclusions

In conclusion, the PVC MPs contained metal additives and especially Pb on a high percentage mass basis. Upon entry of PVC into freshwater, Pb was released from the MPs into aqueous solution. The released Pb was bioavailable in zebrafish and caused an upregulation in expression of *mt2*. Acid-washed PVC MPs caused no changes in gene expression in zebrafish and these data indicate, albeit via aqueous exposure in an early-life stage fish, that the PVC MPs themselves are not toxic. Future studies with MPs must analyse for the presence of chemical additives and impurities in plastics prior to attributing any toxicity observed to the MPs themselves.

Finally, the concentrations of PVC used in the experiments exceed those found in the field by at least several orders of magnitude (Desforges et al., 2014; Phuong et al., 2016). However, the Pb released from MPs into freshwater after 24 h represented a small fraction of the acid-leachable Pb in the PVC. The implications of these data to the environmental exposure of aquatic animals, are that the PVC MPs may remain a longer-term source for Pb and upon entering the acidic environment of the stomach may release a bioavailable dose of Pb. Restrictions on the use of Pb in PVC have been proposed in the EU due to the human and environmental hazards Pb presents, however import of PVC containing Pb compounds to the EU from other jurisdictions is expected to continue thereafter (European Chemicals Agency, 2016). Furthermore, PVC has a lifespan of 50–100 years in industrial and consumer applications, including PVC pipes used to transport potable water. The continuing release of PVC to the environment is therefore expected and fragmentation of PVC into microscale particles may lead to Pb exposure in aquatic animals.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

David Boyle: Conceptualization, Methodology, Investigation, Writing - original draft, Visualization. **Ana I. Catarino:** Methodology, Writing - review & editing. **Nathaniel J. Clark:** Investigation, Writing - review & editing. **Theodore B. Henry:** Conceptualization, Writing - review & editing, Funding acquisition.

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Appendix A. Supplementary data

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